

University of California Office of the President Special Research Programs	<i>Annual Progress Report</i> SUMMARY OF SCIENTIFIC PROGRESS Form 3
<div style="display: flex; justify-content: space-between;"> (Check one) <input checked="" type="checkbox"/> Breast Cancer Research <input type="checkbox"/> Tobacco-Related Disease Research <input type="checkbox"/> Universitywide AIDS Research </div>	
<div style="display: flex; justify-content: space-between;"> AWARD NUMBER: <u>4JB-0119</u> PROJECT YEAR (Check one): <input type="checkbox"/> 1st <input type="checkbox"/> 2nd <input type="checkbox"/> 3rd <input checked="" type="checkbox"/> Final </div>	
PRINCIPAL INVESTIGATOR(S): <u>Martha R. Stampfer (PI) and Paul Yaswen (Co-PI)</u>	
PROJECT TITLE: <u>Genes Involved in Immortalization of Human Mammary Cells</u> INSTITUTION: <u>Ernest Orlando Lawrence Berkeley National Laboratory (LBNL)</u>	
<p>The summary must include:</p> <ul style="list-style-type: none"> a) A statement of each specific aim, followed by an account of progress made towards its accomplishment, including a summary of experimental results. b) If an original aim was dropped or modified, an explanation of the reason for such a change. c) If a new aim was added, progress toward its achievement. d) The relevance of any modified or new specific aim(s) to the project's mission and research priorities <p>Do not exceed 5 pages; number any additional pages as 3a, 3b, etc.</p> <p>(Collaborative Grants should submit one combined progress report.)</p> <p>(See Sections 7.3 and 7.4 for special TRDRP and UARP reporting requirements.)</p> <p>The specific aims of this proposal were designed to understand the genetic changes that permit normal finite lifespan human mammary epithelial cells (HMEC) to overcome replicative senescence and attain an indefinite lifespan. To accomplish this, we originally proposed to use the genetic suppressor element technology to randomly inactivate potential immortalization genes, identify the inactivated gene(s) responsible for suppressing immortality, and use this information to generate new immortal lines. However, while preparing to perform these long-term experiments, we began some short-term experiments examining senescence arrest in our HMEC cultures. These studies proved so fruitful that we redirected our effort to this work.</p> <p>These studies were stimulated by previous reports that expression of oncogenic Raf-1 or Ras in finite lifespan human or rodent fibroblasts causes a growth arrest phenotypically similar to senescence, whereas the same oncogenes overexpressed in some immortal cell lines could induce further markers of malignant transformation. We decided to investigate the effect of Raf-1 in our series of finite lifespan and immortally transformed HMEC. Our initial aims were to determine:</p> <ol style="list-style-type: none"> (1) Whether oncogenic Raf affected our post-selection finite lifespan HMEC. These cells have long-term growth potential associated with down-regulation of the cyclin dependent kinase inhibitor (CKI) p16. The original studies of the effect of Raf had postulated that the induction of the senescence arrest was mediated by p16. Later studies suggested that in some cell types (human and rodent) the arrest could be mediated by changes in expression levels of the CKI p21. Since post-selection HMEC don't express p16, we did not know if it would still growth arrest. (2) Whether immortally transformed HMEC can escape this Raf-induced growth arrest, and if this depends on the method of immortalization. Growth arrest in response to oncogenic Raf or Ras is presumed to serve as a tumor-suppressor mechanism in normal cells. The loss of this response may contribute to malignant progression. (3) The molecular mechanism underlying Raf-induced growth suppression in HMEC, and the ways by which these mechanisms might be altered during transformation. <p>To perform these studies, we utilized a retroviral vector containing an insert encoding oncogenic Raf-1 fused to the hormone-binding domain of the estrogen receptor. The resulting Raf-1 fusion protein is inducible by the estrogen analog 4-hydroxy-tamoxifen (4-HT). To quantify the induced growth suppression, we (a) determined</p>	

the labeling index (LI) and (b) obtained cell counts for cells induced with 4-HT for 6 days. We further analyzed the expression of a variety of molecules that might be involved in Raf-induced arrest, and the effects of overexpression or inhibition of key molecules on growth arrest.

Over the course of our investigations, we have produced the following results:

(1) Oncogenic Raf-1 induces growth-arrest in finite lifespan HMEC. Both post-selection HMEC, which do not express the CKI p16, and pre-selection HMEC, which can express p16, are growth arrested. However, the means by which this arrest is enforced differs significantly from what has been reported in rodent and other human cell types.

Arrested, post-selection HMEC have a flattened, enlarged morphology, but no increased expression of the senescence associated marker, SA- β -gal. They remained p16(-), indicating that p16 can not mediate this arrest. Neither uninduced nor 4-HT-induced cells expressed the CKI p57, and levels of the CKIs p21, p27, and p15 were unchanged. p53 levels decreased and the nearly undetectable levels of p14^{ARF} remained unchanged. Expression of cyclin D1 increased. Levels of the cell cycle regulator cdc25A, and cell survival regulators Bcl-2 and Bad were unchanged. Expression of the viral oncogenes SV40T, HPV16-E6 and -E7, and E1A did not abrogate the arrest, indicating that the Raf-induced arrest in HMEC was not dependent upon either p53 or RB function. This result differs from rodent cells, where the arrest is p53-dependent. Overexpression of c-myc did not affect the Raf-induced growth inhibition.

TUNEL assays showed no evidence of apoptotic cell death, and indicators of metabolic activity suggested a viable cell arrest. Arrested cells expressed lower overall pRB levels, but most of the pRB present was phosphorylated. This result was consistent with assays showing a much greater reduction in cell number than LI. These results suggested that the induced arrest was not simply in G1. Flow cytometry confirmed that cells arrest in S and G2/M as well as in G1.

We tried to determine whether oncogenic Raf-1-induced growth suppression occurred through activation of MEK (MAPK/ERK kinase), a downstream target of Raf-1, by using the MEK inhibitor PD98059, however the concentrations required for MEK inhibition were growth inhibitory by themselves.

We recently assayed pre-selection HMEC, since they are capable of p16 expression, and found that the Raf-induced growth arrest in these cells was associated with **decreased** p16 expression and no change in p21 levels. In contrast, fibroblasts isolated from the same breast tissue growth arrested in the presence of oncogenic Raf with increased p16 and p21 expression, as well as increased expression of SA- β -gal.

Altogether, our data indicate the possibility that additional mechanisms of Raf-induced growth arrest exist beyond those described for rodent cell systems and for other human cell types. This information is important for the possible future design of therapeutic interventions. It also points to the importance of using specifically human mammary epithelial cells to examine how human breast cells in vivo might react to this oncogenic stimulus and to design clinical approaches for activating this pathway in vivo.

These studies have eliminated most of the obvious growth suppressors as instrumental in Raf-induced inhibition. Thus we have not fully succeeded in our aim of understanding the underlying mechanisms. Future studies will need to explore various key elements in the Ras-Raf pathways, and other possible effectors, to determine the mechanism of this arrest in normal human epithelial cells. This information could be instrumental in attempts to invoke this pathway in breast cancer cells, including those lacking functional p53.

(2) The tumor-suppressive response to oncogenic Raf is lost during the process of conversion. Because many immortalized cell lines respond to oncogenic Ras or Raf-1 by transforming, rather than growth arresting, we explored the response of immortalized HMEC to oncogenic Raf-1. These HMEC have been immortalized in our laboratory following exposure to a chemical carcinogen, and/or inactivation of p53, overexpression of ZNF-217 - a putative human breast cancer oncogene, and ectopic introduction of hTERT, the catalytic subunit of the human telomerase complex.

Recent studies from our lab have indicated that even after overcoming replicative senescence, the resultant p53(+) HMEC acquire indefinite proliferative *potential*, but must progress through further changes before exhibiting telomerase activity and uniform good growth. When the mean TRF declines to <3 Kb, these conditionally immortal HMEC undergo a very gradual process, which we have termed conversion, that reactivates telomerase activity and cause other changes in the cells phenotype. Post-selection p16(-) HMEC immortalized by transduction of hTERT, and pre-conversion populations of conditionally immortal HMEC line 184A1 transduced with hTERT, **bypass** the conversion process. However, introduction of hTERT into 184A1 once conversion has begun does not prevent the conversion process from proceeding.

We have found that all our immortal HMEC lines examined that have fully undergone conversion have abrogated their growth inhibitory response to oncogenic Raf. These lines maintain growth, and may acquire additional malignancy-associated properties such as growth factor- and anchorage-independent growth. However immortal lines that did not undergo conversion remain growth inhibited. For example, early passage cells (conditionally immortal, pre-conversion) of the clonally derived HMEC line 184A1 were growth inhibited by oncogenic Raf similar to finite lifespan HMEC, whereas late passage fully converted 184A1 maintained growth. hTERT transduction of **pre**-conversion 184A1 produced uniformly good-growing immortal cells that remained growth-inhibited by Raf, whereas transduction of 184A1 **in**-conversion (cells proceed through conversion) produced uniformly good-growing immortal cells that had abrogated Raf-induced inhibition. hTERT immortalized post-selection HMEC, which did not undergo conversion, also remained growth-inhibited.

These results suggest that the key Ras/Raf signal transduction pathway is significantly altered as a consequence of undergoing conversion, but not simply from overcoming senescence or expressing telomerase activity. As part of the molecular changes that occur during conversion, oncogenic Raf switched from being growth-suppressive to malignancy promoting. We do not yet have a full understanding of the molecular changes that occur during conversion. We know that telomerase is activated, leading to acquisition of TGF β resistance, and that there are changes in expression of c-myc and certain CKIs. Future studies are aimed at unraveling the numerous inter-related changes that occur during conversion, to determine cause and effect relationships. Knowing that the Raf pathway also changes during this period may help provide clues towards this goal.

These studies are also significant in pointing out the very different responses to oncogenic Raf by finite lifespan vs. most immortally transformed cells. The Ras/Raf network is crucial to many aspects of cellular function, and is widely studied. Many studies use immortally transformed cells, purportedly to understand how this pathway functions. However, such transformed cell do not behave the same as normal cells, and use of transformed cells in these studies may present an inaccurate picture of the normal functioning of this crucial cellular network.

Understanding how cancer cells acquire the capacity to be immortal and to abrogate normal tumor-suppressive mechanisms may serve both to increase our understanding of breast cancer progression, and to provide new targets for therapeutic intervention. Our results indicate that normal HMEC have novel means of enforcing a Raf-induced growth arrest and that this tumor suppressive function is lost at a specific stage in malignant transformation. Further studies to elucidate the ways by which immortal, converted HMEC escape this arrest may provide a more complete model of breast carcinogenesis as well as ways to intervene in that process.

This work has been written up and submitted to *Oncogene*:

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Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion